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Quantitation of Small Colony Trifluorothymidine-Resistant Mutants of L5178Y/TK+/- Mouse Lymphoma Cells in RPMI-1640 Medium

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16. ABSTRACT

The L5178Y/TK t/- mouse lymphoma assay quantitates the induction of thymidine kinase deficient (TK-/-) mutants. When plated in Fischer's medium for leukemic cells of mice, mutants appear morphologically as large and small trifluorothymidine-resistant (TFT) colonies; mutants iolated in Fischer's medium using 1 ug/ml TFT are TK deficient and TFT-resistant upon rechallenge with TFT. Cytogenetic analysis has revealed that mutants represent chromosomal mutants of chromosome 11 , whereas mutants appear to represent single gene mutations. A number of laboratories have preferred to use RPMI-1640 medium for the L5178Y/TKt/-assay instead of Fischer's medium. Recent reports by Amacher et al. Because quantitation of the mutants may be an important advantage of the L5178Y/TKt/-mouselymphoma assay, we wished to determine whether it is possible to detect and accurately quantitate TK-deficient mutants in RPMI-1640 medium.

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Quantitation of small colony trifluorothymidine-resistant mutants of L5178Y/TK$^{+/-}$ mouse lymphoma cells in RPMI-1640 medium

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The L5178Y/TK$^{+/-}$ mouse lymphoma assay (Clive and Spector, 1975; Clive et al., 1979) quantitates the induction of thymidine kinase deficient (TK$^{-/-}$) mutants. When plated in Fischer's medium for leukemic cells of mice, mutants appear morphologically as large (λ) and small (σ) trifluorothymidine-resistant (TFT^r) colonies (Moore-Brown and Clive, 1979; Moore-Brown et al., 1981). σ mutants isolated in Fischer's medium using 1 μg/ml TFT are TK-deficient and TFT-resistant upon rechallenge with TFT (Clive et al., 1979; Moore-Brown et al., 1981; Moore et al., 1982). Cytogenetic analysis (Hozier et al., 1981) has revealed that σ mutants represent chromosomal mutants of chromosome 11 (the location of the TK gene in mice [Kozak and Ruddle, 1977]) whereas λ mutants appear to represent single gene mutations.

A number of laboratories have preferred to use RPMI-1640 medium for the L5178Y/TK$^{+/-}$ assay instead of Fischer's medium. Recent reports by Amacher et al. (1979) and Amacher and Paillet (1981) have questioned the ability of non-heat-inactivated RPMI-1640 medium to quantitate σ TK-deficient mutants.

Because quantitation of the σ mutants may be an important advantage of the L5178Y/TK$^{+/-}$ mouse lymphoma assay (Clive et al., 1979, 1980; Clive and Moore-Brown, 1979; Moore-Brown and Clive, 1979; Hozier et al., 1981; Moore-Brown et al., 1981; Moore et al., 1982), we wished to determine whether it is possible to detect and accurately quantitate TK-deficient σ mutants in RPMI-1640 medium.

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Abbreviations: λ, large colony (TFT-resistant, TK-deficient); MMS, methyl methanesulfonate; σ, small colony (TFT-resistant, TK-deficient); TFT, trifluorothymidine; TFT^r, TFT-resistant; TFT^s, TFT-sensitive; THMG, thymidine + hypoxanthine + methotrexate + glycine; TK, thymidine kinase.

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Materials and methods

Cells and media

The $TK^{+/-}$-3.7.2C heterozygote of L5178Y mouse lymphoma cells (Clive and Voytek, 1977) was used.

Fischer's liquid (1×) medium for leukemic cells of mice (Gibco) or RPMI-1640 without HEPES buffer (1×) (Gibco) was used. Each medium was supplemented with sodium pyruvate, antibiotics (Penn-Strep) (Clive and Spector, 1974), and pluronic F68 (Clive et al., 1979). Cells were grown in suspension in medium supplemented with 10% horse serum (Gibco) and cloned in medium supplemented with 20% horse serum and 0.37% agar.

To determine if failure to heat-inactivate RPMI-1640 medium might affect its ability to quantitate σ mutants, we heat-inactivated the Fischer's medium (Clive and Spector, 1975) but tested the RPMI-1640 medium both with and without heating at 57 °C for 45 min.

Chemicals

TFT was obtained from Sigma (St. Louis, MO). Methyl methanesulfonate (MMS) was obtained from Eastman Organic Chemicals (Rochester, NY).

Mutagen treatment and mutant selection

$TK^{+/-}$-3.7.2C cells were treated with the mutagen for 4 h according to combined published procedures (Clive and Spector, 1975; Clive et al., 1979). Cells were treated in medium containing 10% horse serum at a density of 0.6×10^6 cells/ml. $TK^{-/-}$ mutants were selected after 2-day expression using 1 μg/ml (Moore-Brown et al., 1981) or 4 μg/ml TFT. Both 1 and 4 μg/ml TFT were used because 1 μg/ml TFT has been reported to be insufficient for stringent mutant selection with unheated RPMI-1640 medium (Amacher et al., 1979).

Two different protocols were followed. (1) The experiment was performed entirely (i.e., cell treatment, mutant expression, and mutant selection) in each of the 3 different media: heated Fischer's medium, heated RPMI-1640, or unheated RPMI-1640. Under this protocol, $TK^{+/-}$-3.7.2C cells were cleansed (the week prior to treatment) of $TK^{-/-}$ spontaneous mutants using thymidine + hypoxanthine + methotrexate + glycine (THMG) (Clive and Spector, 1975) and Fischer's medium. These cells were then divided into and allowed to adjust to heated RPMI-1640 or unheated RPMI-1640, or left in Fischer's medium for 4 days prior to treatment. (2) In the alternate protocol, cells were treated and mutants were allowed to express in Fischer's medium. Mutant selection was then performed in either Fischer's medium, heated RPMI-1640, or unheated RPMI-1640. THMG cleansing of spontaneous $TK^{-/-}$ mutants was performed in Fischer's medium the week before treatment. All plates were incubated for 9–11 days prior to counting.

Colony counts and sizing were performed on an Artek Model 880 Automatic

Colony Counter as described elsewhere (Moore-Brown and Clive, 1979) using settings which maximize σ mutant detection without producing a spurious background.

Stability of TFT-resistant phenotype

Following treatment protocol (1) (above), 56 σ mutants were isolated from plates cloned in either heated or unheated RPMI-1640 and either 1 or 4 μg/ml TFT. In the same experiment, 14 and 15 spontaneous σ mutants were isolated in unheated RPMI-1640 using 1 or 4 μg/ml TFT, respectively. These cells were grown in nonselective medium for approximately 1 week and then tested in suspension culture for the TFT^r phenotype. Cell cultures were diluted to an approximate density of 6×10^4 cells/ml, divided into two parts, and tested after 2 days for relative increases in cell numbers with and without TFT. For each test, a $TK^{+/-}$ culture was used as a control to demonstrate the ability of TFT to completely arrest cell growth in TK-competent cells.

Results and discussion

In heat-inactivated Fischer's medium the σ phenotype results from the slower growth rate (doubling time > 12 h) of the σ mutant cells. Our experience indicates that $TK^{+/-}$-3.7.2C cells grow at faster rates and form slightly larger colonies in RPMI-1640 medium than in Fischer's medium (data not shown). This growth rate difference probably results from the presence of more nutrients in RPMI-1640 medium (Fischer and Sartorelli, 1964; Moore et al., 1967). The present study was undertaken to determine if σ mutants grow as slowly in the richer RPMI-1640 medium as they do in Fischer's medium or if they grow at a more normal rate (doubling time < 12 h). Such near-normal growth of σ mutants might impair resolution of σ and λ mutants. Furthermore, we wished to determine if σ colonies isolated in RPMI-1640 medium were, in fact, stable TFT-resistant mutants.

Experimental results are shown in Figs. 1 and 2 and Table 1. Neither the frequency nor the relative proportion of σ and λ TK-deficient MMS-induced mutants was significantly affected by any of the media conditions tested. There was no significant difference in results between experiments conducted completely (i.e., from treatment through mutant selection) in each different medium (Fig. 1, Table 1) and those in which mutants were induced and expressed in Fischer's medium, then selected in the different media conditions (Fig. 2). Both approaches were used to determine and separate possible effects of growing σ and λ progenitor mutants in the different media during the expression period and of growing the fully expressed σ and λ mutants into colonies in the different cloning media.

Because the overall total mutant frequencies and the size distribution curves appear to be similar for the 3 media conditions (Fig. 1, Table 1), we conclude that

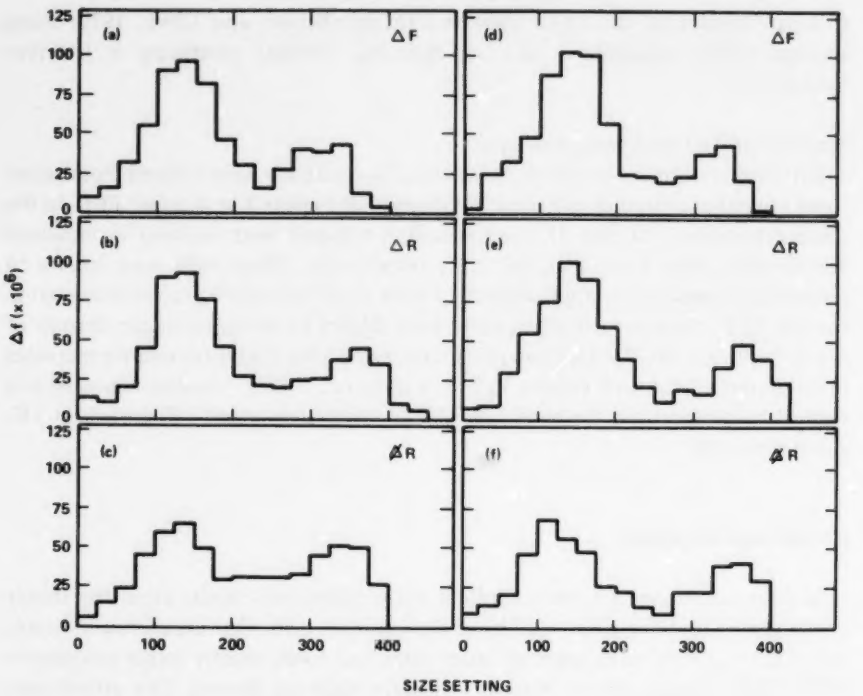

Fig. 1. Size distribution of TFT-resistant mutants following treatment of $TK^{+/-}$-3.7.2C cells with 15 μg/ml MMS. σ colony peak (left); λ colony peak (right). Cell treatment, mutant expression (2 days), and mutant selection were performed in either heated Fischer's medium for leukemic cells of mice (△F), heated RPMI-1640 medium (△R), or unheated RPMI-1640 medium (⌀R). 1 μg/ml (a–c) or 4 μg/ml (d–f) TFT was used for selection. Survivals and total induced mutant frequencies ($\times 10^{-6}$) (calculated according to Clive and Spector, 1975) were as follows: (a) 23%, 527; (b) 28%, 576; (c) 26%, 400; (d) 23%, 557; (e) 28%, 627; (f) 26%, 400.

there is no significant difference in average relative σ and λ mutant growth rates. (In Fischer's medium we have shown that the slow growth of σ mutants results in a decline in the quantitated mutant frequency at increasing times post-treatment [Moore and Clive, 1982].) If σ mutants grow faster in RPMI-1640 medium than in Fischer's medium, one would expect to see both a higher total frequency and less or no decline in frequency with increasing time post-treatment in RPMI-1640 medium. The only consistent difference observed after cloning σ and λ mutants in the 3 media is a slight increase in the average size of the λ mutants in RPMI-1640 medium, seen in Figs. 1 and 2 as a slight rightward shift (larger size setting) of the λ colony peak.

There is no significant difference in total mutant frequencies obtained using heated and unheated RPMI-1640 medium. In Fig. 1, the unheated RPMI-1640

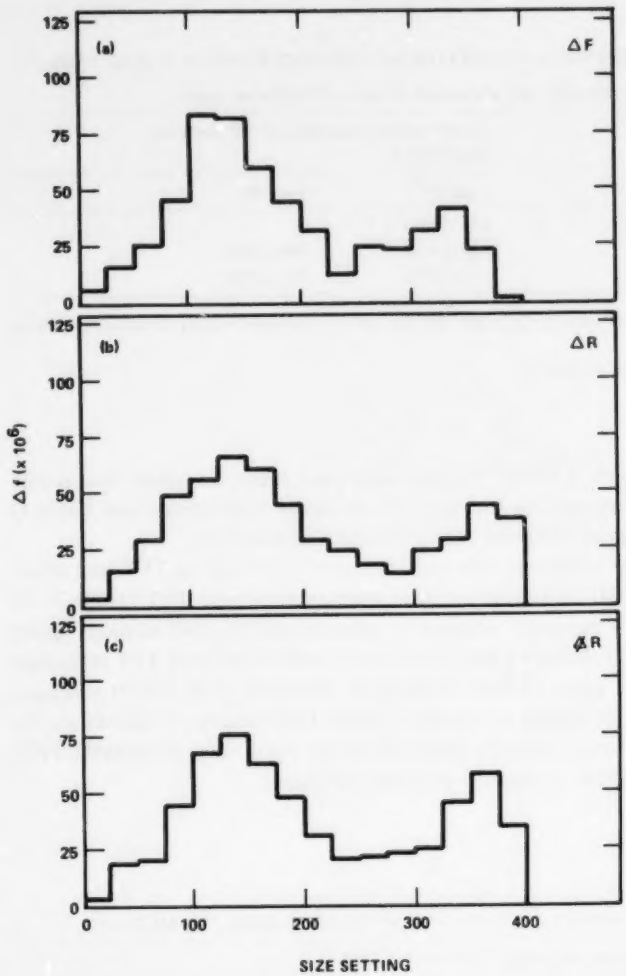

Fig. 2. Size distribution of TFT-resistant mutants following one treatment of $TK^{+/-}$-3.7.2C cells with 15 μg/ml MMS. σ colony peak (left); λ colony peak (right). Cells were treated and mutants allowed to express (2 days) in Fischer's medium for leukemic cells of mice. Mutants were selected using 1 μg/ml TFT in either heated Fischer's medium for leukemic cells of mice (△F), heated RPMI-1640 medium (△R), or unheated RPMI-1640 medium (⧸R). Survival (calculated according to Clive and Spector, 1975) was 31% (based on suspension and cloning growth in Fischer's medium). The total induced mutant frequencies ($\times 10^{-6}$) were as follows: (a) 514; (b) 524; (c) 577.

TABLE 1

TOTAL MUTANT FREQUENCY ($\sigma + \lambda$) FOLLOWING TREATMENT WITH 15 μg/ml MMS

Treatment, expression, and selection were performed in each of 3 different media.

Medium	Total mutant frequency per 10^6 survivors (survival)[a]	
	1 μg/ml[b]	4 μg/ml
Heated Fischer's medium	628 (24%)	–
Heated RPMI-1640	505 (29%)	448 (29%)
Unheated RPMI-1640	475 (23%)	531 (23%)

[a] Survival calculated by the method of Clive and Spector (1975); expressed relative to untreated control cells.

[b] Concentration of TFT used in selection.

medium appears to give a lower mutant frequency than the other two media conditions; however, this pattern was not seen in repeat experiments (see Table 1) and is within the variation observed for MMS-induced mutation.

All 56 MMS-induced σ mutants selected in either 1 or 4 μg/ml TFT and either heated or unheated RPMI-1640 were resistant when retested with TFT (Table 2). In contrast, spontaneous σ 'mutants' isolated in unheated RPMI-1640 medium (using either 1 or 4 μg/ml TFT) showed a high degree of instability of their TFT resistance (Table 3). These results agree with the findings of Amacher et al. (1979) and raise serious questions as to the ability of unheated RPMI-1640 medium to quantitate the σ spontaneous mutant frequency. The reason(s) for the occurrence of unstable TFT^r variants in unheated RPMI medium is presently unknown.

TABLE 2

TFT RESISTANCE OF 56 INDUCED σ MUTANTS ISOLATED IN RPMI-1640 MEDIUM

TFT concentration used in selection (μg/ml)	Percentage of growth with/without TFT	
	Heat-inactivated[a]	Not heat-inactivated
1	101.5 ± 11.6[b] (14)[c] (86–121)[d]	101.5 ± 10.8 (15) (90–134)
4	103.1 ± 8.9 (14) (80–109)	106.5 ± 19.4 (13) (86–157)

[a] Condition of the RPMI-1640 medium used for the selection.

[b] Mean ± S.D. (number of mutants).

[c] Number of mutants tested.

[d] Range.

TABLE 3

TFT RESISTANCE OF 29 SPONTANEOUS σ MUTANTS ISOLATED IN UNHEATED RPMI-1640 MEDIUM

TFT concentration used in selection (μg/ml)	Percentage of growth with/without TFT		TFT^s (%)
	TFT^r	TFT^s	
1	97.3 ± 4.9[a] (6)[b] (85–106)[c]	2.6 ± 1.2 (8) (1.5)	57
4	101.6 ± 11.7 (11) (80–122)	1.5 ± 0.6 (4) (1–2)	27

[a] Mean ± S.D.
[b] Number of mutants tested.
[c] Range.

In Fischer's medium, only 4 of the 56 (7%) spontaneous σ mutants isolated were not resistant to TFT upon retesting. All 55 of the induced σ mutants isolated in Fischer's medium were resistant to TFT when retested following 1 week's growth in nonselective medium (Moore-Brown et al., 1981; Moore et al., 1982).

It is apparent from these experiments that both Fischer's medium and RPMI-1640 medium can be used to quantitate both total mutant frequency ($\sigma + \lambda$) and σ mutant frequency. The difficulty in quantitating the spontaneous σ mutant frequency in unheated RPMI-1640, however, compromises the utility of this medium for routine testing. Since Fischer's medium provides a cleaner selection of σ spontaneous mutants, we recommend Fischer's medium for use with the L5178Y/$TK^{+/-}$ mouse lymphoma assay.

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